Ion-Exchange Reactions between Acid Mucopolysaccharides and Various Cations

By J. R. DUNSTONE

Department of Biochemistry, University of Queensland, Brisbane, Australia

(Received 19 March 1962)

In studies of cation-binding by acid mucopolysaccharides and the possible significance of these reactions in calcification processes, it has been shown, in cartilage, that the chondroitin sulphate component is solely responsible for the cation-binding capacity, that the cation-binding reactions are of the ion-exchange type and that different cations exhibit varying degrees of affinity for the cartilage (Boyd & Neuman, 1951; Dunstone, 1959, 1960).

Cations have been shown to inactivate, *in vitro*, the calcification of rachitic-bone sections (Sobel, 1955). The order of inactivating power of these cations differs from their order of affinity for cartilage (Dunstone, 1960). These differences may be due either to the chondroitin sulphate of the cartilage differing structurally or in its combination with protein from that in the calcifying tissue used by Sobel (1955), or it may be that the process of inhibition of calcification does not involve simple cation-exchange competition. The cartilage preparations so far used represent particular cases. Samples taken from different sources or subjected to different treatments might yield a different order of cation affinity.

Consequently, a study, under a variety of experimental conditions, of the association of these cationic inhibitors of calcification with a number of structurally different sulphated acid mucopolysaccharides (chondroitin sulphate A, chondroitin sulphate B, heparin, heparin monosulphate) in solution and with cartilage-bound chondroitin sulphate has been undertaken. The factors that influence the interaction between cations and acid mucopolysaccharides have been investigated and an attempt has been made to give a quantitative interpretation to the association phenomena. The affinities of the cations for the acid mucopolysaccharides have been measured by the ion-exchange methods outlined by Schubert (1952) and by Djurfeldt & Samuelson (1957*a*, *b*), and have been recorded in the form of exchange parameters calculated from Rothmund & Kornfeld (1918) equations.

EXPERIMENTAL

The interaction of cations with an acid mucopolysaccharide in solution may be treated, as a first approximation, by considering the polyelectrolyte as a phase

separate from the outside solution (see Djurfeldt & Samuelson, 1957*b*), with its electrical charge neutralized by counterions held in the potential field of the macro-ion. The relative affinities of various cations for the polyelectrolyte can then be estimated in an operational fashion by describing the ion distributions obtained when cations from the solution phase exchange with the counterions of the polyelectrolyte phase. Such ion distributions can be described by the empirical relationship of Rothmund & Kornfeld (1918).

For the exchange between a bivalent cation from the solution phase and a univalent cation from the polyelectrolyte phase, the relevant equation is

$$\frac{X_N}{X_M^2} = K \left(\frac{[N]}{[M]^2} \right)^p \quad (1)$$

where X_N and X_M are respectively the equivalent-fractions (for definition see Dunstone, 1960) of the bi- and univalent cations N and M in the polyelectrolyte phase, $[N]$ and $[M]$ are their equivalent concentrations in the solution phase, and K and p are empirical parameters. In this study, the parameter K has been used as a measure of the affinity of cations for the polyelectrolytes. Under specified experimental conditions the parameter p has been found to approximate to unity (see Fig. 1), and this value has been used in the derivation of all subsequent equations.

Schubert (1952) has shown that, when one of the cations under investigation is present at tracer level, the uptake of the tracer cation by the exchange material is proportional to the tracer concentration in the outside solution. The proportionality factor, called the distribution coefficient, K_d , is defined by

$$K_d = \frac{\% \text{ of cation bound to exchanger}}{\% \text{ of cation in solution}} \times \frac{v}{m} = \lambda \times \frac{v}{m} \quad (2)$$

where v is the volume of the solution (l.), m the mass of the exchanger (g.) and λ is the ratio of the percentage of cation bound to that in solution.

Under conditions where a bivalent tracer cation is exchanging for a univalent cation in the polyelectrolyte phase, the equivalent-fraction of the univalent ion in the polyelectrolyte phase (X_M) approximates to unity irrespective of the amount of tracer cation exchanged. Hence, assuming that $X_M = 1$ and $p = 1$, equation (1) can be combined with equation (2) to give

$$K = \frac{K_d}{C} \times [M]^2 \quad (3)$$

where C is the equivalent binding capacity of the polyelectrolyte (equiv./g.).

This equation is also applicable when the polyelectrolyte is combined to a separate insoluble phase, i.e. the acid mucopolysaccharide bound to an insoluble matrix such as cartilage.

Table 1. *Calculation of the parameter K from ion-distribution experiments carried out according to method 1*

Method 1 is described in the Experimental section. Sodium veronal buffer (4 ml.; I 0.16, pH 7.2–7.4), 0.15 g. of Dowex 50 (Na^+ form) resin (0.482 m-equiv.), a ml. of CaCl_2 soln. (53.3 mm), b ml. of chondroitin sulphate A soln. (CSA-2B, 53 mm in disaccharide repeating unit, 0.16 M in NaCl, Na^+ counterion) and $36 - (a + b)$ ml. of NaCl soln. (0.16 M) were mixed and equilibrated for 3 hr. at 20° . The resin was separated from the solution, washed and analysed for its constituent cations. All results are expressed as mean values obtained from duplicate experiments. The ion distributions in the absence of chondroitin sulphate A (calibration of resin) are shown in (A), and the ion distributions in the presence of chondroitin sulphate A in (B).

(A) *Ion distributions in the absence of chondroitin sulphate A*

a (ml.)	4.0	3.2	2.4	1.6	0.8
Ca^{2+} bound to resin (m-equiv.)	0.208	0.189	0.153	0.106	0.060
Equivalent-fraction Ca^{2+} in resin	0.432	0.392	0.317	0.220	0.125
Concn. of Ca^{2+} in soln. (m-equiv./l.)	5.460	3.803	2.571	1.614	0.632
Concn. of Na^+ in soln. (m-equiv./l.)	149.2	151.9	154.2	156.3	158.3
$[\text{Na}^+]^2/[\text{Ca}^{2+}]$	4.08	6.07	9.25	15.1	39.8

(B) *Ion distributions in the presence of chondroitin sulphate A*

a (ml.)	4.0	4.0	4.0	4.0	4.0
b (ml.)	24.0	20.0	16.0	8.0	4.0
Ca^{2+} bound to resin (m-equiv.)	0.147	0.152	0.167	0.174	0.187
Equivalent-fraction Ca^{2+} in resin	0.305	0.315	0.346	0.361	0.388
$[\text{Na}^+]^2/[\text{Ca}^{2+}]^*$	9.7	9.3	8.0	7.3	6.1
Total cation in soln. (m-equiv.)	6.186	6.186	6.186	6.186	6.186
Free Ca^{2+} in soln. (m-equiv.)	0.096	0.100	0.115	0.126	0.150
Ca^{2+} bound to chondroitin sulphate A (m-equiv.)	0.183	0.174	0.144	0.126	0.089
Chondroitin sulphate A in soln. (m-equiv.)	2.544	2.120	1.696	1.272	0.848
Equivalent-fraction Ca^{2+} in chondroitin sulphate A (X_{Ca})	0.072	0.082	0.085	0.099	0.105
$X_{\text{Ca}}/X_{\text{Na}}$	0.084	0.097	0.101	0.122	0.131
K (calculated from equation 1)	0.81	0.90	0.80	0.89	0.80

* From calibration graph.

The distribution of a cation between the polyelectrolyte phase and the solution phase can be measured by maintaining in contact with the aqueous polyelectrolyte solution a separate insoluble phase, i.e. an ion-exchange resin that contains the cation but not the polyelectrolyte (Djurfeldt & Samuelson, 1957*a, b*; Schubert, 1952; Hughes & Klotz, 1956), and determining the distribution of the cation between solution and the ion-exchange resin in the presence and absence of the polyelectrolyte. From these distributions the degree of association of the cation with the polyelectrolyte can be calculated. As the methods of calculation depend on whether equation (1) or (3) is used to describe the ion distributions, details of the relevant calculations are included with the descriptions of the methods actually used for the determination of the ion distributions.

Method of determining ion distributions between acid mucopolysaccharides in solution and cations (method 1)

Procedure. Sodium veronal buffer (approx. one-tenth of total solution volume), NaCl soln. and the chloride solution of the bivalent ion under investigation were mixed and the Na^+ form of the ion-exchange resin was added (0.02–0.5 g.). The mixtures were shaken at 20° for 3 hr. on a rotary shaker (11 rev./min.). After this time the resin was separated from the solution by rapid filtration with a small sintered-glass filter. The resin was washed with water and the bound cations were eluted with 3*N*-HCl. The eluate was analysed for its constituent cations.

A series of similar experiments was carried out with predetermined volumes of acid mucopolysaccharide solution replacing equal volumes of NaCl soln.

Calculations. In the first series of experiments the experimentally determined amount of the bivalent ion in the resin, together with a knowledge of the amounts of the materials taken, were sufficient data for the calculation of the equivalent-fractions in the resin phase and the equivalent concentrations in the solution phase of both the uni- and bi-valent cations. A calibration curve was then drawn by plotting $X_{\text{M}}^2/X_{\text{N}}$ against $[M]^2/[N]$, where X_{M} and X_{N} are respectively the equivalent-fractions of the uni- and bi-valent ions in the resin phase and $[M]$ and $[N]$ are respectively their equivalent concentrations in the solution phase.

The experimentally determined amount of the bivalent cation in the resin, when mucopolysaccharide was present, enabled the ratio $X_{\text{M}}^2/X_{\text{N}}$ to be calculated and the value of $[M]^2/[N]$ read from the calibration graph. The concentrations of the free and mucopolysaccharide-bound cation were then determined and the equivalent-fractions of the cations in the polyelectrolyte phase calculated. The exchange parameters were then calculated by applying equation (1). In these calculations the change in volume of the outer solution caused by the presence of resin and acid mucopolysaccharide has not been taken into consideration. Errors in the calculations arising from these assumptions are thought to be insignificant (see Discussion). Table 1, containing a typical set of experimental details, is included to illustrate the calculations by this method.

Method of determining ion distributions between acid mucopolysaccharides in solution and cations present at tracer levels (method 2)

Procedure. Sodium veronal buffer (approx. one-tenth of total solution volume) containing tracer levels of the cation under investigation, a predetermined volume of acid mucopolysaccharide solution (Na^+ counterion) and NaCl soln. were mixed and shaken for 1 hr. at 20° . Solutions, in which the acid mucopolysaccharide solution was replaced by NaCl soln., were also included. Portions (1–5 ml.) of the solutions were taken for radiochemical analysis and the Na^+ form of the ion-exchange resin (0.02–0.5 g.) was added. The mixture was equilibrated in the manner described in method 1. Portions (1–5 ml.) of the supernatant solutions were then taken for radiochemical analysis. Where no radioactive isotope was available, e.g. Mg^{2+} , or when balance studies were made, the cation contents of both the resin phase and the solution phase were determined.

Calculations. From these experiments the percentage ratios for the uptake of tracer cation by the ion-exchange resin in the presence and the absence of the polyelectrolyte were calculated.

The distribution coefficient for the uptake of the tracer cation by the acid mucopolysaccharide can be represented by

$$K_d = (\lambda_0/\lambda - 1) \times (v/m) \quad (4)$$

where λ_0 and λ are respectively the percentage ratios of the cation bound to the resin to that free in solution in the absence and presence of the acid mucopolysaccharide (see Schubert, 1956). Substitution of equation (4) in equation (3) gives

$$K = \frac{(\lambda_0/\lambda - 1)}{[C]} \times [M]^s \quad (5)$$

where $[C]$ is the equivalent binding capacity of the acid mucopolysaccharide in equiv./l.

The parameter K was then calculated by substituting the experimentally determined percentage ratios in equation (5). Table 2, containing a typical set of experimental details, is included to illustrate the calculation by this method.

Method of determining ion distributions between acid mucopolysaccharides bound to an insoluble phase and cations present at tracer levels (method 3)

Procedure. Sodium veronal buffer (approx. one-tenth of total solution volume) containing tracer levels of the cation under investigation, a predetermined mass of Na^+ -saturated cartilage (0.1–0.5 g.) and NaCl soln. were mixed and equilibrated as described in method 1. Portions (1–5 ml.) of the supernatant solutions were then taken for radiochemical analysis.

Calculations. From these experiments the distribution

Table 2. Calculation of the parameter K from ion-distribution experiments carried out according to method 2

Method 2 is described in the Experimental section. Sodium veronal buffer (2 ml.; I 0.16, pH 7.2–7.4) containing $^{89,90}\text{Sr}$, a ml. of chondroitin sulphate A soln. (CSA-2B, 59 mm in disaccharide repeating unit, 0.16M in NaCl, Na^+ counterion) and $14 - a$ ml. of NaCl soln. (0.16M) were mixed and shaken for 1 hr. at 20° . Three portions (2.0 ml.) were taken for radiochemical analysis and 0.02 g. of Dowex 50 (Na^+ form) resin was added. The solution was equilibrated for 3 hr. at 20° and three portions (2.0 ml.) of the supernatant solution were taken for radiochemical analysis. All results are expressed as the mean values of duplicate experiments.

a (ml.)	Concn. of chondroitin sulphate (m-equiv./l.)	Activity of $^{89,90}\text{Sr}$ (counts/min.)		Distribution of $^{89,90}\text{Sr}$ between phases (%)		λ	$\lambda_0/\lambda - 1$	K (calc. from equation 5)
		Before resin added	After resin added	Soln.	Resin (calc.)			
0	0	1268	365	28.8	71.2	2.47 (λ_0)	—	—
1.0	7.38	1230	434	35.3	64.7	1.83	0.35	1.22
2.0	14.76	1053	430	40.8	59.2	1.45	0.70	1.22
3.0	22.14	1045	482	46.1	53.9	1.17	1.11	1.29
4.0	29.52	1072	523	48.8	51.2	1.05	1.35	1.17

Table 3. Calculation of the parameter K from ion-distribution experiments carried out according to method 3

Method 3 is described in the Experimental section. Samples of Na^+ -cartilage (0.79 m-equiv./g.) were equilibrated with 5 ml. of sodium veronal buffer (I 0.16, pH 7.2–7.4) containing $^{89,90}\text{Sr}$ and 45 ml. of NaCl soln. (0.16M) for 3 hr. at 20° . Three portions (2.0 ml.) of the supernatant solution were taken for radiochemical analysis. All results are expressed as mean values of duplicate experiments.

Mass of cartilage (g.)	$^{89,90}\text{Sr}$ activity (counts/min.)	Distribution of $^{89,90}\text{Sr}$ between phases (%)		$10^3 K_d$ (calc. from equation 2)	K (calc. from equation 3)
		Soln.	Cartilage (calc.)		
0	766	—	—	—	—
0.2	646	84.3	15.7	4.65	1.51
0.5	521	68.0	32.0	4.71	1.53
0.8	456	59.5	40.5	4.26	1.38
1.0	418	54.6	45.4	4.16	1.35

coefficients for the uptake of tracer cation by the bound acid mucopolysaccharide were calculated directly by applying equation (2). Equation (3) was then used to calculate the parameter K . Table 3, containing a typical set of experimental details, is included to illustrate the calculations by this method.

Analytical methods

Uronic acid. The carbazole method of Dische (1947) was used with D-glucuronic acid (Sigma Chemical Co.) as standard.

Nitrogen. This was determined by a micro-Kjeldahl procedure (Francis, Mulligan & Wormall, 1954*b*).

Hexosamine. Determinations were carried out by the procedure of Elson & Morgan (1933) as modified by Boas (1953) with the omission of the ion-exchange separation. Hexosamine was liberated from the acid mucopolysaccharides by hydrolysis with 6*N*-HCl in sealed ampoules at 100° for 15 hr. D-Glucosamine hydrochloride (Nutritional Biochemicals Corp.), submitted to the same treatment, was used as standard.

Sulphate. Determinations were carried out by refluxing the acid mucopolysaccharide with 2*N*-HCl for 4 hr. and then using the method of Fritz, Yamamura & Richard (1957).

Radiochemical analyses. Portions (1–5 ml.) of radioactive solutions were transferred to 25 mm. diameter planchets and 20 drops of an asbestos suspension (Francis, Mulligan & Wormall, 1954*a*) were added to assist spreading, and the planchets were dried under an infrared lamp (80°). Solutions containing ^{140}Ba were freed of the ^{140}La 'daughter' by the method described by Overman & Clark (1960). However, the ^{140}Ba was not precipitated as the sulphate but planchets were prepared from the ^{140}La -free solution in the manner described above. The radioactivities of the ^{45}Ca , $^{89,90}\text{Sr}$ and ^{140}Ba were then measured with an end-window Geiger-Müller tube (no. 18506, Philips Electrical Industries), and the radioactivity of ^{133}Ba samples was measured with a scintillation detector (PW 4111, Philips Electrical Industries), both types of detector being coupled with a standard scaling and recording unit. Amounts of radioactivity were selected so that coincidence corrections were unnecessary (count rate less than 2000/min.). No corrections were necessary for physical decay or self-absorption as measurements of initial and final concentrations were made at approximately the same time from samples of almost identical composition. Samples containing $^{89,90}\text{Sr}$ were left for at least 15 days and those containing ^{140}Ba for 10 days before counting to enable radioactive equilibrium to be attained. All planchets were prepared in triplicate and the activities of the replicates did not differ from the mean value by more than 2% when 4000 or more counts were taken.

Cations. Sodium and potassium were analysed directly by a flame-spectrophotometric method with a Beckman model DU flame spectrophotometer. Magnesium, calcium, strontium and barium were determined by a method described by Dunstone & Payne (1959) or by a modification of this procedure in which all concentrations were decreased to one-fifth and extinctions (at 225 $m\mu$) measured in 5 or 10 cm. cells. In some experiments where magnesium and calcium were present at concentrations greater than 0.1 mM, the magnesium was also determined by the Thiazol Yellow method described by Sandell (1950),

the extinctions (at 535 $m\mu$) being measured in 5 cm. cells, and the calcium was determined by titration with EDTA according to the method of Dunstone (1957).

Physical methods

Viscosity. The terminology for the viscosity functions used was that proposed by Cragg (1946). The measurements were made in modified Ubbelohde viscometers which permitted successive dilutions within the instruments (Davis & Elliott, 1949). The instruments were calibrated with water and sucrose solutions and the necessary corrections for kinetic energy were calculated by the method of Johnson & Rowe (1961). Because of the smallness of the determined corrections (< 0.5%) they have not been applied to the data reported. All solutions were prepared in 0.15*M*-sodium phosphate buffer, pH 7.0, 0.2*M* in NaCl, and centrifuged at 50000*g*_{av.} for 1 hr. (Spinco model L centrifuge, rotor no. 30) before use. The temperature of the system was maintained at 30.0 ± 0.01°, the time of flow was measured to 0.1 sec. and at least six readings were made at each concentration. The intrinsic viscosity $[\eta]$ was determined by plotting reduced viscosity, η_{sp}/c , against c (concentration, g./100 ml.) and extrapolating to $c = 0$.

Optical rotation. Measurements were made on 1.5–2.0% (w/v) solutions in 2 dm. tubes in a Hilger polarimeter. Ten readings were taken on each solution.

Materials

Cartilage. Cation-saturated cartilage was prepared and analysed by the methods described by Dunstone (1959).

Acid mucopolysaccharides. Table 4 lists the sources, methods of preparation and chemical analyses of the acid mucopolysaccharides used. In general, the analytical data reported were sufficient for the identification of the various mucopolysaccharides. The solubility in aqueous ethanol of the calcium salts of all chondroitin sulphate A preparations was tested to verify that these materials contained only minimal amounts of chondroitin sulphate C. In all cases, more than 90% of the precipitable material was recovered at or below ethanol concentrations of 40% (v/v). This indicates contamination with only small quantities of chondroitin sulphate C, which requires higher concentrations of ethanol for precipitation (see Meyer, Davidson, Linker & Hoffman, 1956).

Unless otherwise stated, acid mucopolysaccharides for use in binding experiments were prepared by dissolving approx. 2 g. of the acid mucopolysaccharide in 100 ml. of water and passing the solution three times through a column of Dowex 50 (Na^+ form). The solutions were concentrated under reduced pressure and clarified by centrifuging at 23000*g*_{av.} for 1 hr. (Spinco model L centrifuge, rotor no. 21). The pH was adjusted to 7.2–7.4 (NaOH or HCl), NaCl was added to give the desired I and a crystal of thymol added to prevent bacterial growth. Solutions were kept refrigerated until used. In some experiments K^+ ion was substituted for the Na^+ ion in all steps of the preparation.

Ion-exchange resin for equilibrium experiments. Dowex 50 (X 8; 50–100 mesh) was prepared in various cation forms by the method of Schubert (1952). Analysis of the air-dried Na^+ form showed a capacity of 3.37 m-equiv./g. and a moisture content of 29.0% (dried at 110° to constant weight). The capacity varied in the range 3.37–3.21 m-equiv./g. over a period of 2 years.

Veronal buffer (pH 7.2–7.4, *I* 0.16). Diethylbarbituric acid (28.6 m-moles) (Drug Houses of Australia Ltd.) was dissolved in about 200 ml. of a solution containing 28.6 m-moles of NaOH; 151.4 m-moles of NaCl and 20 m-moles of HCl were then added and the solution was diluted to 1 l. In experiments with a tracer cation about 0.1 mc of the

radioactive isotope was added per litre of buffer. In experiments where the Na⁺ ion was replaced by other cations, the oxide or hydroxide of the particular cation was substituted for the NaOH and its chloride for the NaCl in amounts to give a total *I* of 0.16.

Radioactive isotopes. The radioactive isotopes ⁴⁵Ca, ⁸⁵Sr,

Table 4. *Sources, methods of preparation and properties of acid mucopolysaccharides used in distribution experiments*

All values given in this Table have been corrected for the loss in weight on drying.

Substance	[α] _D (c 1.5–2.0 in water)	Percentages				Loss in wt. on drying (110° <i>in vacuo</i> over P ₂ O ₅ for 2 hr.)	Protein (non- hexosamine N × 6.25)
		Hexosamine	Uronic acid (carbazole)	N	SO ₄		
Chondroitin sulphate A [Na ⁺ counterion; L. Light and Co. Ltd. (CSA-1)]	– 26	30.4	31.6	3.3	17.0	19.6	5.7
Chondroitin sulphate A [unknown counterion; General Biochemicals Inc.; as received (CSA-2A)]	– 32	32.2	33.4	3.9	14.7	7.9	8.6
Chondroitin sulphate A [K ⁺ counterion; General Biochemicals Inc.; purified twice by method of Einbinder & Schubert (1950) (CSA-2B)]	– 22	27.9	37.0	2.6	16.4	20.1	2.5
Chondroitin sulphate A [K ⁺ counterion; prepared from acetone-dried bovine nasal septa by the method of Dorfman & Cifonelli (1957); purified once by method of Einbinder & Schubert (1950) (CSA-3)]	– 27	29.0	33.7	2.6	15.8	4.0	2.0
Chondroitin sulphate A [Na ⁺ counterion; prepared from acetone-dried bovine nasal septa by method of Mathews & Dorfman (1953); purified by precipitating twice with Co(NH ₃) ₆ ³⁺ ions (CSA-4)]	– 31	25.4	30.5	4.1	14.5	11.0	13.1
Chondroitin sulphate A [Na ⁺ counterion; prepared from fresh bovine nasal septa by method of Mathews & Dorfman (1953); purified by precipitating twice with Co(NH ₃) ₆ ³⁺ ions (CSA-5)]	– 32	25.6	33.7	3.8	13.3	18.0	10.3
Partially desulphated chondroitin sulphate A [Na ⁺ counterion; prepared from purified chondroitin sulphate A (General Biochemicals Inc.) by method of Kantor & Schubert (1957) (CSA-6)]	– 18	36.0	44.5	2.8	11.1	16.2	—
Chondroitin sulphate B [β -heparin, Na ⁺ counterion; F. Hofmann-La Roche and Co. Ltd.; prepared from ox lung by method of Marbet & Winterstein (1953)]	– 66	28.5	18.2	2.6	17.2	14.5	2.3
Heparin I [Na ⁺ counterion; Abbott Laboratories Ltd.]	+ 50	26.0	44.4	2.3	33.8	21.7	1.6
Heparin II [Na ⁺ counterion; The Upjohn Co.]	+ 48	24.0	44.8	2.6	39.6	16.5	4.4
Heparin monosulphate [Na ⁺ counterion; The Upjohn Co.; purified by fractionation with cetylpyridinium chloride by method of Cifonelli & Dorfman (1960); the 'heparin monosulphate' fraction so obtained was then refractionated by the same method]	+ 51	28.2	47.1	2.6	20.7	16.8	2.4

^{133}Ba and ^{140}Ba were obtained as their chlorides from the Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A. The ^{45}Ca and ^{133}Ba had specific activities of 35 384 mc/g. and 0.804 mc/g. respectively, whereas the ^{89}Sr , which contained some ^{90}Sr (< 10% of original activity), and the ^{140}Ba were carrier-free.

RESULTS

Preliminary experiments

Time of attainment of equilibrium. In all experiments equilibrium was reached within 1 hr., and no changes in the ion distributions occurred in the subsequent 25 hr.

Validity of methods. (i) Cation balances were carried out on all equilibria involving acid mucopolysaccharides in solution. The recovery of added bivalent cation determined by analysis of the solution and resin phases was found to be $101 \pm 3\%$. The capacity of the cation-exchange resin as determined by the analysis of the resin after equilibration was $99.3 \pm 1.5\%$ of the value obtained before equilibration.

(ii) When radioactive isotopes were used experiments were carried out to check whether the isotope was adsorbed on to the walls of the glass vessels. Radioactive solutions were transferred from vessel to vessel with 30 min. shaking between each transfer. Radiochemical analyses were made before each transfer and no significant changes in count rate occurred in five transfers with any of the isotopes used.

(iii) Equations (2), (3) and (4) are valid only when the distribution coefficients are independent of the extent to which the tracer cation is adsorbed (Schubert, 1956). The concentrations of tracer cation at which the distribution coefficients should begin to deviate were calculated by the method described by Schubert (1956). All experiments were carried out at concentrations less than the calculated figures. At constant I , the distribution coefficients were found to be constant for all concentrations of tracer cation used, except with cartilage, where differences were observed when the tracer concentration approached the maximum allowable (for further details see below, and Discussion section).

(iv) In checking the reliability of experiments with radioactive isotopes Schubert (1952) has shown that a plot of $1/\lambda$ versus $[C]$ should give a straight line, which when extrapolated to $[C] = 0$ gives an intercept of $1/\lambda_0$. This plot has been used in all the systems studied and the values of λ_0 deduced in this way were found to be $101 \pm 6\%$ of the values obtained by direct measurement.

An estimate has been made of the accuracy of determinations of the parameter K by calculating the coefficients of variation (s.d./mean value). The values for methods 1, 2 and 3 (Experimental

section) were respectively 0.19 (14), 0.08 (59) and 0.14 (12), where the figures in parentheses represent the number of degrees of freedom.

Reversibility of exchange reactions. The reversibility of the reactions with the ion-exchange resin and with cartilage was checked by measuring the distribution coefficients over a range of v/m values. The distribution coefficients were found to be independent of the v/m ratio (for cartilage, see Table 3) and therefore the reactions can be considered reversible (Schubert, 1956).

The reversibility of reactions with acid mucopolysaccharides in solution was investigated by varying the order of addition of the reagents. The resulting ion distributions were independent of the sequence in which the reagents were added and hence these reactions may also be considered reversible (Djurfeldt & Samuelson, 1957*a, b*).

Evaluation of the parameter p . The parameter p can be evaluated by applying equation (1) to the results of ion distributions obtained by method 1 (Experimental section), and plotting $\log X_M^2/X_N$ against $\log[M]^2/[N]$, the slope of the straight line obtained being equal to p . Such an experiment has been carried out with Ca^{2+} ions and chondroitin sulphate A (CSA-2B, Na^+ counterion) at I 0.023 and I 0.16. The straight lines plotted in Fig. 1 have been calculated by the method of least squares (Yule & Kendall, 1945) and their slopes are respectively 0.98 ± 0.04 (s.d.) and 0.91 ± 0.03 (s.d.).

Affinities of cations for acid mucopolysaccharides in solution

The distributions between tracer cations and various acid mucopolysaccharides in solution have been determined at pH 7.2–7.4 by methods 1 and 2 described in the Experimental section. As the affinity of K^+ ions for the acid mucopolysaccharides could not be determined directly, experiments were carried out with K^+ ions instead of Na^+ ions as the univalent exchanging ions. A comparison of the affinities of a given bivalent ion for the Na^+ - and for the K^+ -acid mucopolysaccharide shows which of the two univalent ions is more strongly held by the polyelectrolyte. Owing to the fact that heparin samples show varying degrees of sulphation two heparin preparations have been studied. Shortage of material prevented a more detailed study of the family of compounds which assay as heparin monosulphate. The values for the parameter K have been calculated from the relevant equations and are tabulated in Table 5.

These results show marked differences in the relative affinities of the cations for the various acid mucopolysaccharides. Because of these wide variations subsequent studies have been concerned with the factors that may affect these cation affinities.

Effect of dialysing solutions before experiment on the affinity of cations for acid mucopolysaccharides

Owing to the fact that a marked difference in cation-binding affinity for Ca^{2+} ion was shown by

Fig. 1. Evaluation of the parameter p of equation (1). Ion-distribution experiments were carried out between Ca^{2+} ions and chondroitin sulphate A (CSA-2B, Na^+ counterion) by method 1 (Experimental section) at pH 7.2–7.4 and I 0.023 and 0.16. The terms $X_{\text{Na}}^2/X_{\text{Ca}}$ and $[\text{Na}^+]^2/[\text{Ca}^{2+}]$ of equation (1) were calculated (see Table 1) and their logarithms plotted as indicated in the Figure. ●, I 0.023; ○, I 0.16. The straight lines were calculated by the least-squares method and the slopes were respectively 0.98 ± 0.04 (s.d.) and 0.91 ± 0.03 (s.d.).

the two samples of heparin, the effect of dialysing all samples in the initial stage of preparation, to remove possible small-molecular-weight substances, was investigated. This process produced significant changes in cation affinity and in the relative affinities for the various cations only with heparin 1 (Abbott Laboratories Ltd.). A second sample of heparin from the same manufacturer showed similar properties to the first sample. For comparison the results of the dialysis experiments with heparin 1 have been included in Table 5.

Effect of desulphation of chondroitin sulphate A on its affinity for cations

To investigate the nature of the cation association a comparison has been made of the affinity for Ca^{2+} ions of chondroitin sulphate A (CSA-2B) and desulphated chondroitin sulphate A (CSA-6) at I 0.16, pH 7.2–7.4. The results (Table 6) show clearly that the amount of cation bound is decreased considerably by sulphate removal, as shown by the large reduction in the value of $\lambda_0/\lambda - 1$, but the cation affinity remains unaltered.

Affinity of cations for different chondroitin sulphate A preparations

The exchange parameters for reactions between Ca^{2+} ions and a number of chondroitin sulphate A preparations have been determined with method 2 (Experimental section). Analytical details of the preparations studied are given in Table 4. The intrinsic viscosity has been used as an index of the molecular weight of the various samples. A low-molecular-weight sample was prepared by incubating 4 ml. of a solution of CSA-2B (Na^+ counterion, I 0.16), 2 ml. of veronal buffer (I 0.16, pH 7.2–7.4) containing ^{45}Ca , 10 ml. of NaCl soln. (I 0.16)

Table 5. Calculated parameters for exchange reactions between acid mucopolysaccharides and cations

Ion-distribution experiments (pH 7.2–7.4) were carried out according to method 2 (Experimental section) unless otherwise specified. The parameters, K , have been calculated from equation (5) and the results are quoted as mean values with the numbers of determinations in parentheses. Values obtained at I 0.16 are given in (A), and values obtained at I 0.06 are given in (B).

Cation originally associated with acid mucopolysaccharide		Exchanging cation	Chondroitin sulphate A (CSA-2B)	Chondroitin sulphate B	Heparin 1 (Abbott)	Heparin 1 (Abbott) dialysed	Heparin 11 (Upjohn)	Heparin mono-sulphate
(A)	Na^+	Mg^{2+}	0.59 (3)	1.05 (3)	—	1.49 (3)	—	0.87 (2)
		Mg^{2+}	0.4 (9)*	—	—	—	—	—
		$^{45}\text{Ca}^{2+}$	0.75 (10)	0.90 (8)	148 (4)	3.32 (4)	3.24 (2)	1.8 (2)
		Ca^{2+}	0.84 (5)*	—	—	—	—	—
		$^{89,90}\text{Sr}^{2+}$	1.21 (8)	0.90 (3)	73 (6)	2.29 (7)	—	1.5 (2)
		$^{138}\text{Ba}^{2+}$	1.84 (12)	0.98 (6)	7.3 (6)	2.65 (4)	—	5.4 (2)
(B)	Na^+	$^{45}\text{Ca}^{2+}$	1.18 (5)	1.12 (3)	—	2.52 (3)	—	—
		$^{45}\text{Ca}^{2+}$	0.57 (2)	0.63 (2)	—	2.20 (2)	—	1.4 (2)
		$^{89,90}\text{Sr}^{2+}$	0.78 (2)	0.55 (2)	—	2.32 (2)	—	—
		$^{138}\text{Ba}^{2+}$	1.11 (2)	0.74 (2)	—	2.44 (2)	—	—

* Method 1; K calculated from equation (1) (p 1).

Table 6. *Effect of desulphation of chondroitin sulphate A on the affinity for cations*

Distribution experiments were carried out as described in method 2 (Experimental section) at I 0.16, pH 7.2–7.4. Na^+ ion was the counterion originally associated with the acid mucopolysaccharides and $^{45}\text{Ca}^{2+}$ was the other exchanging cation. Results are expressed as mean values of figures obtained from duplicate experiments.

Preparation	Concn. of acid mucopolysaccharide (moles of disaccharide repeating unit/l.)	Equivalent-binding capacity (equiv./l.)	$\lambda_0/\lambda - 1$	K (calc. from equation 5)
Chondroitin sulphate A (CSA-2B)	0.015	0.030	0.85	0.73
Partially desulphated chondroitin sulphate A (CSA-6)	0.015	0.023	0.66	0.74

containing 750 i.u. of testicular hyaluronidase (Rondase, Evans Medical Supplies Ltd., London) at 37° for 24 hr. The enzyme was inactivated by heating at 100° for 3 min. This solution was then treated in the manner described in method 2 (Experimental section). Preliminary experiments showed that the intrinsic viscosity was approximately halved and the reducing value (determined by the method of Park & Johnson, 1949) approximately doubled by this procedure. Neither the presence of inactivated enzyme nor heating of the solution altered the value of the exchange parameter.

The results of these experiments (Table 7) show that (a) the protein component, in samples of relatively low molecular weight, has little effect on the exchange parameter, (b) degradation also causes little change in the exchange parameter, and (c) samples (CSA-5, CSA-4) extracted by mild procedures, which contain chondroitin sulphate of high molecular weight plus some protein, have greater affinity for Ca^{2+} ions than the other preparations.

This higher cation affinity was investigated further by measuring the relative affinities of samples CSA-5 and CSA-2B for Ca^{2+} , Sr^{2+} and Ba^{2+} ions. The results, given in Table 8, confirm the previous observation (Table 7) that sample CSA-5 has a greater affinity for cations than sample CSA-2B, and also show that the affinities for Ca^{2+} , Sr^{2+} and Ba^{2+} ions, relative to each other, are almost the same with both samples.

The results suggest that the acid mucopolysaccharide component of the preparation is primarily responsible for the binding of cations and that the differences in the magnitude of the exchange parameters is due to the state of polymerization of this component. However, the contribution of the protein component has not been completely evaluated, and it may be of some significance as the protein-containing samples showed viscosity properties quite different from essentially protein-free preparations. The viscosity differences are illustrated in Fig. 2, in which reduced viscosity is plotted against concentration. The slope constants k' [$k' = \text{slope}/[\eta]$, see Mathews

Table 7. *Affinities of various chondroitin sulphate A preparations for Ca^{2+} ions*

Ion-distribution experiments were carried out according to method 2 (Experimental section) at I 0.16, pH 7.2–7.4, with a number of chondroitin sulphate A preparations (see Table 4). In all experiments Na^+ ion was the cation originally associated with the chondroitin sulphate A and $^{45}\text{Ca}^{2+}$ was the other exchanging cation. Intrinsic viscosity has been used as an index of molecular weight. The parameter K was calculated from equation (5). All results are given as mean values obtained from duplicate experiments.

Substance	$[\eta]$	K
Enzymically degraded chondroitin sulphate A	0.19	0.64
CSA-2A	0.26	0.72
CSA-2B	0.38	0.75
CSA-1	0.42	0.66
CSA-3	0.44	0.76
CSA-5	0.77	1.07
CSA-4	1.07	1.6

Table 8. *Relative affinities of Ca^{2+} , Sr^{2+} and Ba^{2+} ions for two chondroitin sulphate A preparations*

Ion-distribution experiments were carried out according to method 2 (Experimental section) at I 0.16, pH 7.2–7.4, with two chondroitin sulphate A preparations of intrinsic viscosities 0.38 and 0.77 respectively (see Table 7). In all experiments Na^+ ion was the cation originally associated with the acid mucopolysaccharide. The parameter K was calculated from equation (5). All results are given as mean values obtained from duplicate experiments.

Exchanging cation	K (calc. from equation 5)	
	CSA-2B ($[\eta]$ 0.38)	CSA-5 ($[\eta]$ 0.77)
$^{45}\text{Ca}^{2+}$	0.75	1.07
$^{89,90}\text{Sr}^{2+}$	1.21	1.57
$^{133}\text{Ba}^{2+}$	1.84	2.99

(1956)] for the protein-containing samples (CSA-2A, CSA-4, CSA-5) are considerably larger than those for the other preparations. This difference is probably due to the presence of the protein and reflects differences in solution properties of protein-free and protein-containing preparations that may influence the exchange parameters.

Effect of ionic strength on the affinity of cations for acid mucopolysaccharides in solution

In the experiments to evaluate the parameter p , the results (Fig. 1) indicated that I had a marked effect on the ion distributions at pH 7.2–7.4. This effect has been studied further by determining the ion distributions between Ca^{2+} ions and chondroitin sulphate A (Na^+ counterion) over a range of I (I 0.013–0.5) with method 2 (Experimental section). The values for the parameter K determined from these experiments are tabulated in Table 9. They show a decrease in magnitude with decrease in I . Owing to this wide variation in K

with I , the relative affinities of the various cations for the acid mucopolysaccharides have been measured at I 0.06, and the relative affinities compared with those obtained at I 0.16. The results, tabulated in Table 5 (B), confirm the decrease in affinity with the decrease in I (Table 9) and show that the relative affinities for chondroitin sulphate A and for chondroitin sulphate B are similar at both I values, with slightly smaller differences for chondroitin sulphate A. However, with heparin at I 0.16 the affinity for Ca^{2+} ions is greater than those for Mg^{2+} , Sr^{2+} and Ba^{2+} ions, but at I 0.06 there is little difference between the affinities for Ca^{2+} , Sr^{2+} and Ba^{2+} ions.

Affinity of cations for cartilage-bound acid mucopolysaccharides

The reactions between cartilage and cations have been examined under conditions similar to those used in studies with the acid mucopolysaccharides in solution (I 0.16, pH 7.2–7.4).

The ion distributions have been measured with method 3 (Experimental section). Values for the parameter K are recorded in Table 10. The order of increasing affinity indicated by the value of K for the cations studied is clearly Ba^{2+} , Ca^{2+} $\approx$ Sr^{2+} .

This order of cation affinity was unexpected, as a previous study (Dunstone, 1960) had shown the order of increasing affinity for cartilage to be Ca^{2+} , Sr^{2+} and Ba^{2+} ions. A possible explanation of this reversal of order is afforded by the fact that the ^{133}Ba used was of low specific activity and hence the actual equivalent-fraction of barium in the

Fig. 2. Reduced viscosity data of various chondroitin sulphate A preparations. Analytical data for the preparations are given in Table 4. Concentrations are based on the weight of the preparations after drying at 110° *in vacuo* over P_2O_5 for 2 hr. The slope constants, k' shown below, have been calculated from the relation $k' = \text{slope}/[\eta]$. $\circ$, CSA-2A (k' 0.7); $\bullet$, CSA-2B (k' 0.1); $\triangle$, CSA-5 (k' 1.7); $\blacktriangle$, CSA-4 (k' 1.8). Straight lines similar to that for CSA-2B were obtained with the other preparations listed in Table 7.

Table 9. *Effect of ionic strength on the affinity of cations for acid mucopolysaccharides in solution*

Ion-distribution experiments were carried out according to methods 1 and 2 (Experimental section) at pH 7.2–7.4 and the parameter K was calculated respectively from equation (1) and equation (5). The acid mucopolysaccharide used in these experiments was chondroitin sulphate A (CSA-2B, Na^+ counterion) and Ca^{2+} was the other exchanging cation. All results are mean values of figures obtained from triplicate experiments.

Method used	I	K
2	0.013	0.09
1	0.023	0.16
2	0.027	0.29
2	0.030	0.31
2	0.047	0.42
2	0.060	0.57
1	0.075	0.61
2	0.097	0.67
2	0.160	0.75
1	0.160	0.80
2	0.180	1.17
2	0.264	1.73
2	0.346	1.80
2	0.520	2.70

cartilage at equilibrium would be larger than the corresponding value for calcium and strontium, which were used in almost carrier-free form. This greater equivalent-fraction could give rise to an affinity constant lower than that obtainable at equivalent-fractions comparable with those of

Table 10. *Calculated parameters for exchange reactions between cartilage-bound acid mucopolysaccharides and cations*

Ion-distribution experiments were carried out according to method 3 (Experimental section) at I 0.16, pH 7.2–7.4. Na^+ -cartilage (0.79 m-equiv./g.) was used in all experiments. The parameter K was calculated from equation (3) and the results are quoted as mean values with the number of determinations in parentheses.

Exchanging cation	K
$^{45}\text{Ca}^{2+}$	1.46 (4)
$^{89,90}\text{Sr}^{2+}$	1.44 (4)
$^{138}\text{Ba}^{2+}$	0.37 (5)
$^{45}\text{Ca}^{2+}$	1.37 (7)*

* Calculated from Boyd & Neuman (1951).

Table 11. *Effect of the tracer-cation concentration on the exchange parameters for reactions between cations and cartilage*

Ion-distribution experiments were carried out according to method 3 (Experimental section) with Na^+ -cartilage (0.79 m-equiv./g.) at I 0.16, pH 7.2–7.4. The parameter K was calculated from equation (3) and the results are expressed as mean values obtained from duplicate experiments. Mass of cartilage 0.5 g., volume of solution 50 ml.

Tracer cation	Initial concn. of tracer cation (mm)	K
$^{45}\text{Ca}^{2+}$	10^{-5}	1.46
Ca^{2+}	0.2	0.36
$^{138}\text{Ba}^{2+}$	0.1	0.37
$^{140}\text{Ba}^{2+}$	$< 10^{-5}$	0.76

Table 12. *Effect of ionic strength on the affinity of cations for cartilage-bound acid mucopolysaccharide*

Ion-distribution experiments were carried out according to method 3 (Experimental section) with Na^+ -cartilage (0.79 m-equiv./g.) at pH 7.2–7.4. The tracer cation was $^{45}\text{Ca}^{2+}$. The parameter K was calculated from equation (3) and the results are expressed as mean values of figures obtained from triplicate experiments.

I	K
0.013	0.43
0.033	0.62
0.076	1.04
0.116	1.25
0.160	1.46
0.216	1.82
0.316	2.60
0.416	6.8

calcium and strontium (see Discussion section). To test this hypothesis, exchange parameters were determined for the reactions $\text{Ca}^{2+} + \text{Na}^+$ -cartilage and $\text{Ba}^{2+} + \text{Na}^+$ -cartilage with different concentrations of the tracer cations. The concentrations used were less than those which should produce significant variation in the distribution coefficients (for calculations of these concentrations see Schubert, 1956). The results shown in Table 11 indicate that the exchange parameters are sensitive to changes in the concentration of the tracer cation, an increase in the cation concentration causing a decrease in the magnitude of the exchange parameter. This suggests that the above-mentioned hypothesis is probably correct. However, the order of cation affinity for cartilage, under these experimental conditions, remained unaltered.

Variations in the value of the parameter K for the reaction $^{45}\text{Ca}^{2+} + \text{Na}^+$ -cartilage, over a range of I 0.013–0.416, have been investigated by the same method and the calculated values for this parameter are recorded in Table 12. There is a decrease in K with the decrease in I .

DISCUSSION

Nature of cation association with acid mucopolysaccharides in solution

Gilbert & Myers (1960) have investigated the binding of cations by chondroitin sulphate A, and have concluded that only 'single-site' association between cation and carboxyl group occurs. If this is so, then the influence of the sulphate groups on cation association should be small. Experiments in which some of the sulphate groups of chondroitin sulphate A have been removed indicated that the degree of cation association was markedly decreased by this process, but the affinity (as indicated by the value of the parameter K) of the cation for the acid mucopolysaccharide remained constant (Table 6). The fact that removal of sulphate did not cause any change in the affinity suggests that the binding is mainly of a type not depending on the specific properties of the anionic groups, i.e. probably electrostatic. Specific binding by the carboxyl groups probably occurs, but it is thought that its contribution to the overall cation association, as measured in these experiments, is small.

In view of the wide variation of the exchange parameters with I (Tables 5B and 9) an analysis of the theory of cation interactions with acid mucopolysaccharides has been made to (a) evaluate the factors that may cause the exchange parameter K to vary, and (b) obtain a better understanding of the mechanism of the cation association so that the results of the experiments with the various acid mucopolysaccharides may be validly interpreted.

By considering the polyelectrolyte as a separate phase it is possible to describe the ion-exchange reactions as Donnan membrane equilibria (Overbeek, 1956). If the polyelectrolyte is in equilibrium with an aqueous solution containing a bivalent cation N , a univalent cation M and the anion A , then according to the Donnan concept

$$\begin{aligned} \alpha_M \cdot \alpha_A &= \bar{\alpha}_M \cdot \bar{\alpha}_A \\ \alpha_N \cdot \alpha_A^2 &= \bar{\alpha}_N \cdot \bar{\alpha}_A^2 \end{aligned} \quad (6)$$

where α represents the activity of a component in the solution phase and $\bar{\alpha}$ the activity in the polyelectrolyte phase.

Cancelling the common anion activities and introducing concentrations and activity coefficients in place of activities, we get

$$\frac{[\bar{N}]}{[\bar{M}]^2} = \frac{\bar{\gamma}_M^2}{\bar{\gamma}_N} \cdot \frac{[N] \cdot \gamma_N}{[M]^2 \cdot \gamma_M^2} \quad (7)$$

where γ_N , γ_M and $\bar{\gamma}_N$, $\bar{\gamma}_M$ represent respectively the activity coefficients of the cations in the solution and polyelectrolyte phases, and $[N]$, $[M]$ and $[\bar{N}]$, $[\bar{M}]$ represent respectively the equivalent concentrations in the solution and polyelectrolyte phases.

In terms of equivalent-fractions rather than concentrations for the polyelectrolyte phase

$$\frac{X_N}{X_M^2} = \bar{C} \cdot \frac{\bar{\gamma}_M^2}{\bar{\gamma}_N} \cdot \frac{[N] \cdot \gamma_N}{[M]^2 \cdot \gamma_M^2} \quad (8)$$

where $\bar{C}$ is the total equivalent concentration of the polyelectrolyte phase (expressed as equivalents per unit volume).

Equation (8) and equation (3) are identical when $p = 1$ and

$$K = \bar{C} \cdot \frac{\bar{\gamma}_M^2}{\bar{\gamma}_N} \cdot \frac{\gamma_N}{\gamma_M^2} \quad (9)$$

Thus the variation of K with I can arise from the variation of the terms on the right-hand side of equation (9). As polyelectrolytes in solution swell as I is lowered (Hermans & Overbeek, 1948) the value of the term $\bar{C}$ would be expected to decrease owing to the larger volume occupied by the polyelectrolyte, and this lowering of concentration would probably cause an increase in the magnitude of the ratio $\bar{\gamma}_M^2/\bar{\gamma}_N$. The ratio γ_N/γ_M^2 would not be expected to vary by more than 10–15% over the range of I studied. These estimates have been made with the activity-coefficient data given by Harned & Owen (1958). The data at present available do not permit an evaluation of the magnitude of these changes but it would seem unlikely that these factors alone could explain the observed 30-fold variation in K over the range of I studied.

The nature of the distribution of counterions about the polyelectrolyte could, in itself, provide an explanation of these variations in K with I . The

univalent counterions that neutralize the electrical charge of a polyelectrolyte are thought to be distributed throughout the ionic atmosphere of the polyion with a fraction of their number held so close to the polymer skeleton that they may be termed 'bound'; this closely held fraction is visualized as a relatively mobile monolayer, whereas the other counterions, more distant from the polyion, are considered 'free' (Lapanje, Haebig, Davis & Rice, 1961). The so-called 'bound' counterions have been shown to move with the polyion in transport experiments (Huizenga, Grieger & Wall, 1950*a, b*; Mathews, 1959; Strauss, Woodside & Wineman, 1957) and the fraction of the total counterions which are 'bound' varies with I within a fairly limited range. For example, Strauss *et al.* (1957) showed that, with a polyphosphate, the fraction of 'bound' counterions (Na^+) varied over the range 0.4–0.7 as I was varied from 0.01 to 0.35. With bivalent ions the 'bound' fraction is thought to be much larger than that with univalent ions (Aldrich, 1958; Gregor, 1956). Nevertheless, all counterions, both 'free' and 'bound', seem to be immobilized sufficiently by the polyion to prevent them contributing to the I of the solution. The validity of this statement is illustrated by the fact that increases in acid mucopolysaccharide concentration did not alter the exchange parameters. For example, in the reaction $^{45}\text{Ca}^{2+} + \text{Na}^+ - \text{chondroitin sulphate A}$, the value of the exchange parameter remained constant even when the acid mucopolysaccharide concentration was changed from 0.01 to 0.04 equiv./l. at I 0.02. From these considerations, the exchange between a bivalent cation from solution and a univalent cation associated with the acid mucopolysaccharide may be described by two ion distributions, one associated with the 'bound' counterions and the other with the 'free' counterions. As the bivalent ions are predominantly 'bound', the exchange involving 'bound' univalent ions and bivalent ions from solution probably makes a greater contribution to the overall distribution than the exchange with 'free' ions. As the fraction of 'bound' univalent ions is approximately 0.5 with the chondroitin sulphates (Mathews, 1959), it might be expected that for each bivalent ion exchanged, one 'bound' univalent ion is displaced. Thus the exchange parameter for the reaction could be represented by the following modified form of equation (3)

$$K = \frac{K_d}{[C]} \times [M] \quad (10)$$

If the experimental data of Table 9 are fitted to this equation the value of K becomes approximately constant over the range of I studied. This indicates the possible validity of the suggested mechanism for chondroitin sulphate A. A similar set of

conditions should also apply to reactions with chondroitin sulphate B. The changes in K with I do not appear to be as marked with heparin as with the chondroitin sulphates. This could be explained in terms of a greater fraction of 'bound' univalent counterions with heparin, which would be expected because of the higher charge density of this substance. Although this distinction between 'bound' and 'free' counterions is useful in providing a possible explanation of the variations of the parameter K with I , the concept is, to a certain extent, arbitrary.

Two other factors, namely the effect on the calculation of the exchange parameters of the volume of the polyelectrolyte phase and of co-ion penetration into the polyelectrolyte phase, must also be considered.

The dimensions of a polyelectrolyte molecule in solution are often described in terms of its hydrodynamic volume, which approximates to the domain of the molecule in solution. This domain is usually freely permeable to solution, hence it seems probable that the exchange reactions occur in a region close to the polymer skeleton. If this is so then the volume of the polyelectrolyte phase involved in the ion-exchange reactions should be considerably smaller than the hydrodynamic volume and thus small compared with the volume of the outside solution. However, should the volume of the polyelectrolyte phase involved in ion-exchange reactions approach the hydrodynamic volume, with the exclusion of cations other than those required to maintain electrical neutrality, then the true concentration of the solution phase would be greater than the calculated concentration. This would mean that the value of K calculated from equation (1) is greater than the true value. However, the fact that calculated values of K remain constant over fourfold variations in polyelectrolyte concentration (0.25–1.0%, w/v) at I 0.01–0.5 supports the contention that the functional volume of the polyelectrolyte phase is indeed small. Therefore no correction has been applied for the volume of the acid mucopolysaccharides.

The calculation of the equivalent-fractions of the cations associated with the polyelectrolyte is complicated by the fact that co-ions may also be present in the polyelectrolyte phase. These co-ions must be accompanied by an equivalent amount of cation, over and above the amount required to maintain the electrical neutrality of the polyelectrolyte phase. Provided that the concentration of cations in the polyelectrolyte phase is large in comparison with that of the outside solution, the application of the Donnan concept shows that co-ions will be largely excluded from this phase; where there are low concentrations in the polyelectrolyte

phase co-ion penetration may have considerable influence on the calculation of the amounts of cations associated with this phase.

Acid mucopolysaccharides have a high surface potential, and as the functional volume of the polyelectrolytes is probably small, the cation concentration in the polyelectrolyte phase is probably fairly high, and thus co-ion penetration into this phase should be small. However, some co-ions will be present in the polyelectrolyte phase, but as sufficient data are not available to apply a correction, the effect of the penetration of these co-ions has been neglected.

Affinity of cations for acid mucopolysaccharides in solution

The factors that influence the selectivity of exchange materials for cations have been extensively studied (Kitchener, 1957) but no explanation of the selectivity, in terms of a few simple factors, has been forthcoming. However, as the relative affinities of the cations for the various acid mucopolysaccharides have been determined from experiments carried out under identical conditions, it is reasonable to suppose that the observed differences were due to the natures of the various polyelectrolyte molecules in solution.

The chondroitin sulphates A and B are linear polyelectrolytes with identical chain characteristics in dilute solution and the same electrophoretic potentials (Mathews, 1959, 1960). Nevertheless, chondroitin sulphate B shows little selectivity for cations whereas chondroitin sulphate A is selective. This indicates a probable difference in physical configuration in solution between the two types of molecule. This difference obviously arises from the difference in chemical structure between the two compounds, namely the presence of D-glucuronic acid in chondroitin sulphate A and of L-iduronic acid in chondroitin sulphate B, the remaining parts of the molecules being identical (Muir, 1961).

Mathews (1960) has indicated that chondroitin sulphate B binds cations more strongly than does chondroitin sulphate A. The work reported here does not support this, but shows that the order of affinity depends upon the cation used and possibly on the molecular weight of the polyions. Thus it seems unlikely that the differences in cation affinity between these chondroitin sulphates, at least for small cations, can be explained in terms of a single factor, namely electrostatic interaction between neighbouring groups as suggested by Mathews (1960) for the association of the large cation, $\text{Co}(\text{NH}_3)_6^{3+}$.

Wolfson (1958) has indicated that heparin may be composed of disaccharide repeating units containing equimolar amounts of D-glucuronic acid and D-glucosamine with between two and three

sulphate groups per disaccharide. The hexosamine nitrogen and some of the hydroxyl groups are sulphated. Heparin monosulphate (heparitin sulphate) is thought to be a polymer containing repeating units of D-glucosamine disulphate (O- and N-sulphate groups) and D-glucuronic acid, with a sulphate-free portion containing repeating units of N-acetylglucosamine and D-glucuronic acid attached. There is also the possibility that heparin monosulphate has a branched structure (Linker & Sampson, 1960). However, Cifonelli & Dorfman (1962) have indicated that L-iduronic acid is also present in preparations of heparin and heparin monosulphate. From the above considerations it would appear that, although the structures of these two substances have not yet been fully elucidated, there does exist a number of structural similarities between them. This similarity in structure is further illustrated by the fact that both of these substances have cation affinities of comparable magnitude and also show a similar order of cation affinity. The same general order of affinity for the bivalent cations studied has been found with heparin, heparin monosulphate and chondroitin sulphate A, except that with heparin there is a greater affinity for calcium than for the other cations and with heparin monosulphate there is almost an equal affinity for calcium and strontium (the slightly greater affinity for calcium may be due to the presence of some heparin in the heparin monosulphate fraction). This common order of affinity, namely

supports the idea that, in general, the reactions studied are of the ion-exchange type with non-specific electrostatic binding rather than of the more specific chelate type where the order

would be expected (see Martell & Calvin, 1952).

In general, heparin and heparin monosulphate show greater affinity for cations than the chondroitin sulphates. This is probably due to the greater charge density of these polymers produced by the larger number of charged groups per repeating unit (see Boyd, Soldano & Bonner, 1954; Hutschneker & Deuel, 1956). Both the heparin and heparin monosulphate contain N-sulphate groups as well as O-sulphate groups and these N-sulphate groups may exert some specific effects, which are, in part, responsible for the greater affinity.

The chondroitin sulphates have a greater affinity for sodium than for potassium, whereas with heparin the reverse is true (Table 5). This behaviour is probably due to specific effects of the groups responsible for the binding. In the chondroitin sulphates the binding is shared equally by weakly acidic carboxyl groups and strongly acidic O-

sulphate ester groups, whereas with heparin strongly acidic O- and N-sulphate groups predominate over the weaker carboxyl groups. These reactions are similar to those with weakly acidic carboxylic acid resins where sodium is preferred to potassium, and with strongly acidic sulphonic acid resins where potassium is preferred to sodium. The magnitude of the measured exchange parameters indicates that the bivalent ions are bound more strongly than the univalent ions. The calculation upon which this statement is based is made by substituting the experimentally determined value of K and various values of X_N in equation (1) and then calculating the equivalent-fraction of the bivalent ion in the solution phase. The shape of the plot of X_N against the equivalent-fraction of the bivalent ion in the solution phase indicates which of the ions is the more strongly bound (see Dunstone, 1960).

The effect of dialysis of the heparin solutions has been included because of the marked difference in behaviour of the dialysed and non-dialysed samples, and because some workers (Mathews, 1960) have used material from the same source (Abbott Laboratories Ltd.) without indicating any prior treatment. The fact that the high binding affinity and the order of cation affinity were altered by dialysis showed that there was present in the non-dialysed material some small-molecular-weight substance or substances that had a strong affinity for the cations studied. Similar substances were not detected in a sample from a different manufacturer (The Upjohn Co.). The presence of this powerful binding agent or agents did not appear to influence the anticoagulant properties, as all samples tested had similar anticoagulant activities.

Affinity of cations for cartilage-bound acid mucopolysaccharides

The cation affinities for cartilage-bound acid mucopolysaccharide obtained in the present study differ from those obtained by Dunstone (1960) in that the magnitudes of the exchange parameters, measured at the same I , are different [$\text{Ca}^{2+} + \text{Na}^{+}$ -cartilage at $I \approx 0.03$: present study, K 0.62; Dunstone (1960), K 0.05]. This variation can only be attributed to the different experimental conditions used in the two studies. Kitchener (1957) has pointed out that the affinity of cations for exchange materials varies with the cation composition of the exchanger. It has been found that the lower the equivalent-fraction of a particular cation in the exchanger, the higher is the affinity for this particular cation. This, then, could explain (a) the greater affinity for cations observed in the present study, where the equivalent-fractions of the particular cation are very small compared with the relatively large equivalent-fractions obtained in

the earlier work, and (b) the sensitivity of the methods used in the present study to changes in the concentration of the tracer cations (Table 11).

The results of the present study indicate that the cation content of the mucopolysaccharides in solution exerts no effect on the measured exchange parameters, as similar values were obtained for the $\text{Ca}^{2+} + \text{Na}^{+}$ -chondroitin sulphate A and the $\text{Mg}^{2+} + \text{Na}^{+}$ -chondroitin sulphate A systems at equivalent-fractions approaching zero and at equivalent-fractions of approximately 0.1 (Tables 5, 1). This is to be expected, as Kitchener (1957) has indicated that the lower the degree of cross-linking of the exchanger the less the effect of exchanger composition on the exchange parameters, hence no such effect should be observed where a linear polyelectrolyte in solution is concerned, but may be observed in a solid exchanger such as cartilage, where the polyelectrolyte is somewhat restricted.

In cartilage systems the variation of the exchange parameter with I was not as marked as in systems with the acid mucopolysaccharide in solution. The introduction of solution activity coefficients in the manner described by Boyd & Neuman (1951) slightly reduced the variation but the values did not approach the constancy observed by these authors, who studied the distributions only over a narrow range of I . No explanation of the variations can be given at present.

Physiological significance of cation association with acid mucopolysaccharides

The order of the ability of cations to inhibit calcification (Sobel, 1955) is not the same as any of the cation-affinity series obtained with acid mucopolysaccharides either bound or in solution. This may mean that the process of inactivation of calcification *in vitro*, envisaged as selective binding of cations to a 'local factor' such as an acid mucopolysaccharide, is not due to an ion-exchange process of the type reported in this paper. On the other hand, the order of affinity of cations for the calcifying matrix could well be the same as that for inactivation of calcification owing to the effects of (a) the chemical structure of the agent responsible for the binding, (b) the physical state of this agent, i.e. solid, gel or solution, and (c) the cation composition of the matrix, upon the order of affinity.

The suggestion that the cation composition of the matrix may have an important regulatory function in determining cation affinities has not previously been made, but the results show that it may be important. For example, a deficiency of a particular cation in the matrix may enable the matrix to exhibit a greater affinity for this cation than for another cation that would normally have greater intrinsic affinity for the matrix but is present in the matrix in larger quantity.

There might be better agreement between the affinity series and the inactivation series if the binding of some cations were more covalent than electrostatic. It has been suggested that the groups of the acid mucopolysaccharides are predominantly concerned with electrostatic binding and not specific site binding. Thus for covalent binding to occur, the participation of groups other than those present in acid mucopolysaccharides might be expected. However, J. R. van Wazer (in the Discussion on the paper of Harris & Rice, 1954) has pointed out that with certain unifunctional polyelectrolytes both non-specific electrostatic and specific covalent binding occur, even with the alkali metals. Hence, a system that binds cations by one or both of these mechanisms could exist in the systems studied by Sobel (1955), which bear closer resemblance to those found *in vivo* than those studied here, if (a) other functional groups were provided by the matrix to which the mucopolysaccharide is bound and/or (b) the polyelectrolyte is of such a nature as to permit both types of binding. In view of the experimental findings reported in this paper, both of these possibilities seem unlikely.

Owing to the variety of factors that may affect the order of cation affinity for a calcifying matrix, more must be known about the chemical and physical nature of the matrix at the site of calcification before any conclusions can justly be made about the mechanism of cation-binding. Although acid mucopolysaccharides have been thought to provide sites, by cation-binding, for the initiation of crystal nuclei upon which epitactic growth of bone mineral takes place, evidence from the present work indicates that these substances, alone, bind cations in a rather non-specific way by electrostatic forces. Such binding would not be expected to provide specific sites for crystal growth.

SUMMARY

1. The interactions of cations with acid mucopolysaccharides in solution have been treated as ion-exchange reactions.

2. The relative affinities of the cations Na^{+} , K^{+} , Mg^{2+} , Ca^{2+} , Sr^{2+} and Ba^{2+} for chondroitin sulphate A, chondroitin sulphate B, heparin and heparin monosulphate have been measured and recorded in the form of exchange parameters calculated from a Rothmund & Kornfeld (1918) equation.

3. The orders of increasing cation affinity are: K^{+} , Na^{+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} for chondroitin sulphate A; K^{+} , Na^{+} , $\text{Mg}^{2+} \approx \text{Ca}^{2+} \approx \text{Sr}^{2+} \approx \text{Ba}^{2+}$ for chondroitin sulphate B; Na^{+} , K^{+} , Mg^{2+} , Sr^{2+} , Ba^{2+} , Ca^{2+} for heparin; Na^{+} , Mg^{2+} , $\text{Ca}^{2+} \approx \text{Sr}^{2+}$, Ba^{2+} for heparin monosulphate. The differences in the orders of affinity have been attributed to

differences in the structures of the various acid mucopolysaccharides.

4. Exchange parameters have been found to vary with ionic strength, and a theoretical evaluation of the origins of this variation has been made.

5. Under the experimental conditions used, the association of cations with acid mucopolysaccharides in solution is thought to be mainly of an electrostatic type.

6. Mucopolysaccharides extracted from cartilage by mild procedures bind cations more strongly than the materials extracted by relatively harsh procedures. The cause of the stronger binding appears to be associated with the state of polymerization of the mucopolysaccharide, but the effect of the protein which is extracted with the mucopolysaccharide has not been completely evaluated.

7. A comparison of the affinity of cations for acid mucopolysaccharides in solution and for cartilage-bound acid mucopolysaccharide has shown that the order of affinity for cations of the cartilage-bound acid mucopolysaccharide under the experimental conditions described by Dunstone (1960) is similar to that for chondroitin sulphate A in solution. However, when ion-exchange methods with tracer cations are used to study reactions with cartilage, the exchange parameters vary with the cation composition of the cartilage phase and cause the order of affinity for cations to alter. This type of variation does not occur when the acid mucopolysaccharide is present in solution.

8. The exchange parameters for reactions between cartilage and cations vary with ionic strength but the variation is considerably less than that which occurs when the acid mucopolysaccharide is present in solution.

9. The significance of these results with respect to the function of acid mucopolysaccharides in calcification processes is briefly discussed.

I wish to express my thanks to Professor H. J. G. Hines of the University of Queensland for his continued interest and helpful advice, and to Professor A. G. Ogston, F.R.S., of the Australian National University, for his interest and helpful criticism. Thanks are also due to Lesley Power for her skilled technical assistance; to F. Hofmann-La Roche and Co. Ltd., Basle, Switzerland, for gifts of β -heparin; to Abbott Laboratories Ltd., N. Chicago, Ill., U.S.A. for a gift of sodium heparin; to The Upjohn Co., Kalamazoo, Mich., U.S.A. for gifts of sodium heparin and heparin monosulphate; and to Foggitt Jones Pty. Ltd. for supplying the nasal septa.

REFERENCES

- Aldrich, B. I. (1958). *Biochem. J.* **70**, 236.
 Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.
 Boyd, E. S. & Neuman, W. F. (1951). *J. biol. Chem.* **193**, 243.
 Boyd, G. E., Soldano, B. A. & Bonner, O. D. (1954). *J. phys. Chem.* **58**, 456.
 Cifonelli, J. A. & Dorfman, A. (1960). *J. biol. Chem.* **235**, 3283.
 Cifonelli, J. A. & Dorfman, A. (1962). *Biochem. biophys. Res. Commun.* **7**, 41.
 Cragg, L. H. (1946). *J. Colloid Sci.* **1**, 261.
 Davis, W. E. & Elliott, J. H. (1949). *J. Colloid Sci.* **4**, 313.
 Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
 Djurfeldt, R. & Samuelson, O. (1957a). *Acta chem. scand.* **11**, 1209.
 Djurfeldt, R. & Samuelson, O. (1957b). *Acta chem. scand.* **11**, 1216.
 Dorfman, A. & Cifonelli, J. A. (1957). In *Methods in Enzymology*, vol. 3, p. 24. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Dunstone, J. R. (1957). *Med. J. Aust.* **2**, no. 16, 571.
 Dunstone, J. R. (1959). *Biochem. J.* **72**, 465.
 Dunstone, J. R. (1960). *Biochem. J.* **77**, 164.
 Dunstone, J. R. & Payne, E. (1959). *Analyst*, **84**, 110.
 Einbinder, J. & Schubert, M. (1950). *J. biol. Chem.* **185**, 725.
 Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
 Francis, G. E., Mulligan, W. & Wormall, A. (1954a). *Isotopic Tracers*, p. 137. London: The Athlone Press.
 Francis, G. E., Mulligan, W. & Wormall, A. (1954b). *Isotopic Tracers*, p. 277. London: The Athlone Press.
 Fritz, J. S., Yamamura, S. S. & Richard, M. J. (1957). *Analyt. Chem.* **29**, 158.
 Gilbert, I. G. F. & Myers, N. A. (1960). *Biochim. biophys. Acta*, **42**, 469.
 Gregor, H. P. (1956). *Trans. N.Y. Acad. Sci.* **18**, 667.
 Harned, H. S. & Owen, B. B. (1958). *The Physical Chemistry of Electrolytic Solutions*. New York: Reinhold Publishing Corp.
 Harris, F. E. & Rice, S. A. (1954). *J. phys. Chem.* **58**, 733.
 Hermans, J. J. & Overbeek, J. T. G. (1948). *Rec. Trav. chim. Pays-Bas*, **67**, 761.
 Hughes, T. R. & Klotz, I. M. (1956). *Meth. biochem. Anal.* **3**, 265.
 Huizenga, J. R., Grieger, P. F. & Wall, F. T. (1950a). *J. Amer. chem. Soc.* **72**, 2636.
 Huizenga, J. R., Grieger, P. F. & Wall, F. T. (1950b). *J. Amer. chem. Soc.* **72**, 4228.
 Hutschneker, K. & Deuel, H. (1956). *Helv. chim. acta*, **39**, 1038.
 Johnson, P. & Rowe, A. J. (1961). *Biochem. J.* **79**, 524.
 Kantor, T. G. & Schubert, M. (1957). *J. Amer. chem. Soc.* **79**, 152.
 Kitchener, J. A. (1957). *Ion Exchange Resins*, chap. 3. London: Methuen and Co. Ltd.
 Lapanje, S., Haebig, J., Davis, T. & Rice, S. A. (1961). *J. Amer. chem. Soc.* **83**, 1590.
 Linker, A. & Sampson, P. (1960). *Biochim. biophys. Acta*, **43**, 366.
 Marbet, R. & Winterstein, A. (1953). *Helv. chim. acta*, **34**, 2311.
 Martell, A. E. & Calvin, M. (1952). *Chemistry of the Metal Chelate Compounds*, chaps. 5 and 9. New York: Prentice-Hall Inc.
 Mathews, M. B. (1956). *Arch. Biochem. Biophys.* **61**, 367.
 Mathews, M. B. (1959). *Biochim. biophys. Acta*, **35**, 9.
 Mathews, M. B. (1960). *Biochim. biophys. Acta*, **37**, 288.
 Mathews, M. B. & Dorfman, A. (1953). *Arch. Biochem. Biophys.* **42**, 41.

- Meyer, K., Davidson, E. A., Linker, A. & Hoffman, P. (1956). *Biochim. biophys. Acta*, **21**, 506.
- Muir, H. M. (1961). *Symp. biochem. Soc.* **20**, 4.
- Overbeek, J. T. G. (1956). *Progr. Biophys. biophys. Chem.* **6**, 58.
- Overman, R. T. & Clark, H. M. (1960). *Radioisotope Techniques*, p. 330. New York: McGraw-Hill Book Co. Inc.
- Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
- Rothmund, V. & Kornfeld, G. (1918). *Z. anorg. Chem.* **103**, 129.
- Sandell, E. B. (1950). *Colorimetric Determination of Traces of Metals*, p. 425. New York: Interscience Publishers Inc.
- Schubert, J. (1952). *J. phys. Chem.* **56**, 113.
- Schubert, J. (1956). *Meth. biochem. Anal.* **3**, 247.
- Sobel, A. E. (1955). *Ann. N.Y. Acad. Sci.* **60**, Art. 5, 713.
- Strauss, U. P., Woodside, D. & Wineman, P. (1957). *J. phys. Chem.* **61**, 1353.
- Wolfson, M. L. (1958). In *Polysaccharides in Biology, Trans. 4th Conf.*, p. 115. Ed. by Springer, G. F. New York: Josiah Macy, Jun. Foundation.
- Yule, G. U. & Kendall, M. G. (1945). *An Introduction to the Theory of Statistics*, p. 315, London: Griffin and Co. Ltd.

Biochem. J. (1962) **85**, 351

A Comparative Study of Nicotinamide Nucleotide Coenzymes during Growth of the Sheep and Rat

BY P. CAIGER AND R. K. MORTON

Department of Agricultural Chemistry, Waite Agricultural Research Institute, University of Adelaide

AND O. H. FILSELL AND I. G. JARRETT

C.S.I.R.O. Division of Biochemistry and General Nutrition, Adelaide, South Australia

(Received 6 April 1962)

Although observations have been made on the concentrations of nicotinamide nucleotide coenzymes in the tissues of a number of laboratory animals, there appears to be no information on the concentration of the oxidized and reduced forms of these substances in ruminant tissue.

Many aspects of carbohydrate metabolism in the adult sheep differ from those in monogastric animals (see Lindsay, 1959), and differences in enzyme patterns have been shown to exist between lamb, adult-sheep and rat tissues (Jarrett & Filsell, 1958; McLean, 1958*a*; Gallagher & Buttery, 1959). Moreover, the metabolism of a young lamb more closely resembles that of a non-ruminating animal than that of an adult sheep, which relies largely on lower fatty acids for its energy requirements (Jarrett & Potter, 1952, 1953).

The quantitative significance of glucose in the metabolism of mature ruminants and the inter-relationships between glucose and lower fatty acids has still to be elucidated (Lindsay, 1959; Jarrett & Filsell, 1961). Since nicotinamide nucleotide coenzymes are required for both oxidative metabolism and reductive syntheses, further information is required on the supply and demand for these coenzymes in ruminants that use acetate both for oxidative metabolism and for lipogenesis (see Lindsay, 1959; Annison & Lindsay, 1961).

The rate of synthesis and the cellular concentrations of nicotinamide nucleotide coenzymes in

the liver increase considerably during growth of the mouse and rat (Branster & Morton, 1956; Morton, 1958; Dawkins, 1959), and appear to be closely related to the rate of cell division (Morton, 1961). Similar information with respect to ruminants has not been available.

The concentrations of both oxidized and reduced nicotinamide nucleotide coenzymes in the livers of lambs and sheep, and of young and mature rats, have now been measured. The results are discussed in relation to the growth and changes in metabolism of these animals.

EXPERIMENTAL

Enzyme preparations

Isocitrate dehydrogenase. This was prepared from pig heart (Grafin & Ochoa, 1950); the ammonium sulphate fraction obtained between 50 and 60% saturation was dissolved in 0.04M-sodium potassium phosphate buffer, pH 7.4, dialysed against the same buffer for about 8 hr. at 0° and then stored at -15°.

Glutathione reductase. This was prepared from dried peas (Kaplan, Colowick & Neufeld, 1953); the ammonium sulphate fraction obtained between 40 and 60% saturation was dissolved in 0.1M-tris-HCl buffer, pH 7.5, and stored at -15°.

Alcohol dehydrogenase. A 1% (w/v) solution of the crystalline enzyme (C. F. Boehringer und Soehne, Mannheim, Germany) was used.

Enzyme specificity. Under the conditions of assay used